1

Laboratory Studies on Life-span, Growth, Aging, and Pathology of the Annual Fish, *Cynolebias bellottii* Steindachner[1]

Robert K. Liu[2] and Roy L. Walford[3]

(Plates I-III; Text-figures 1-4)

Various aspects of the biology of *Cynolebias bellottii* were investigated to provide baseline information necessary for studies on the physiology of aging. This species has proven to be a suitable animal for gerontological studies. Maintained since hatching at 22 ± 1°C., on an eight hour photoperiod with twice daily feedings of live, adult *Artemia,* males reached 50% mortality at 14.5 months, females at 15.5 months. Factors affecting the life-span of annual fishes are discussed. Growth was most rapid from hatching to two months, after which increases in length and weight continued up to ten months. Beyond these periods, individual fish may either continue to grow, show no increase in growth, or lose weight and/or decrease in length. Population averages for these parameters tended to decline with advanced age. Certain symptoms of pathology increased with age. Emaciation, equilibrium disturbances, and spinal curvature were the major gross symptoms. On the microscopic level, hyperplastic and heterotopic thyroid tissue and gill filament changes were most frequent.

Introduction

A number of teleost fish are thought to be annuals (Myers, 1952). Such fish should be useful experimental animals for gerontologic research providing that their life-spans under laboratory conditions are as short or nearly as short as their designation "annual" implies, and that their husbandry is not overly difficult. We have shown that at 22°C. *Cynolebias adloffi* does indeed have a life-span of approximately one year, also that growth rate and life-span are significantly increased by lowering the ambient temperature (Walford and Liu, 1965; Liu and Walford, 1966). However, *C. adloffi* is prone to infections of *Oodinium,* piscine tuberculosis, and has a high incidence of reticuloendothelial hyperplasia. In the present report we present our initial observations with regard to mortality rates, growth rates throughout life, and gross and microscopic pathology of the related annual, *C. bellottii.* This species is superior to *C. adloffi* as an animal for research on aging, in that it is much more resistant to infection than the latter and does not show a high incidence of idiopathic reticuloendothelial hyperplasia. In addition, research on the biology of this species in semi-natural conditions (Bay, 1966, work in progress) provides data for comparison against laboratory conditions. Our studies indicate that *C. bellottii* displays determinate growth and true senescence under the conditions maintained in our laboratories. At 22°C., and under the feeding schedule specified, they displayed a 50% survival of 14.5 to 15.5 months, with a life table entirely characteristic of a population undergoing senescent change. No fish lived longer than 18 months.

[1]Supported by United States Public Health Service Research Grant No. HD-00534.

[2]Department of Zoology, University of California, Los Angeles, California 90024.

[3]Department of Pathology, University of California School of Medicine, Los Angeles, California 90024.

Material and Methods

Adults were collected in the vicinity of Buenos Aires, Argentina, and sent to our laboratory on 23 October 1963 by Professor Rogelio Lopez. Seven hundred and ninety eggs were collected at the end of two weeks when the last of 37 survivors died (19 males, 18 females out of the total shipment of 97 specimens). Five hundred and sixty eggs were hatched at 22 ± 1°C. on 6 to 8 January 1964 and the viable juveniles were maintained at this temperature in an aquarium 33.0 cm wide x 88.9 cm long x 50.8 cm high, receiving eight hours of light daily. At one month, the population was counted and weighed; 35 individuals were selected as the experimental population and transferred to another tank of equal dimensions. At two months of age, the experimental population was counted, sexed, weighed, and placed in two tanks of the same size; 6 males and 11 females in one, and 6 males plus 12 females in the other. From three months onward, water was exchanged monthly by means of a submerged pump between these separate tanks, in addition to removal and replacement of eight liters of water, in order to equalize conditions in both tanks and to reduce the concentration of growth-inhibiting factors (Rose, 1959a and 1959b; Kawamoto, 1961). For 21 months, the average pH was 5.2 (range 4.0-7.0) and rarely differed by more than 0.4 between tanks. Except for procedures detailed in Walford and Liu (1965), no other means of regulating water quality were employed, since in its native habitat annual fish experience too diverse a range of conditions throughout the seasons (Boschi, 1957; Hoigne and Turner, pers. comm.) to be duplicated in the laboratory. Since the inception of our research on *Cynolebias,* we have encountered no problems with keeping these fish in either a mixture of tap and distilled water or entirely tap water (see Kinne, 1960 for an analysis of tap water from the Los Angeles Aqueduct).

Fish were fed twice daily for 3 to 4 weeks on newly-hatched *Artemia* nauplii, then shifted gradually to a mixed diet of washed tubificid worms and live, adult brine shrimp for two more months, after which the diet was limited to the brine shrimp in order to reduce the possibility of infectious agents being introduced with the food. Enough *Artemia* were introduced daily after the third month to provide approximately one gram per wet weight for each fish.

All techniques on collection and incubation of eggs, weighing and measuring, and autopsies and sectioning of histological material are given in Walford and Liu (1965).

Results

1. *Survival and life-span.* A survival curve was calculated from the mortality data of Table I

Table I

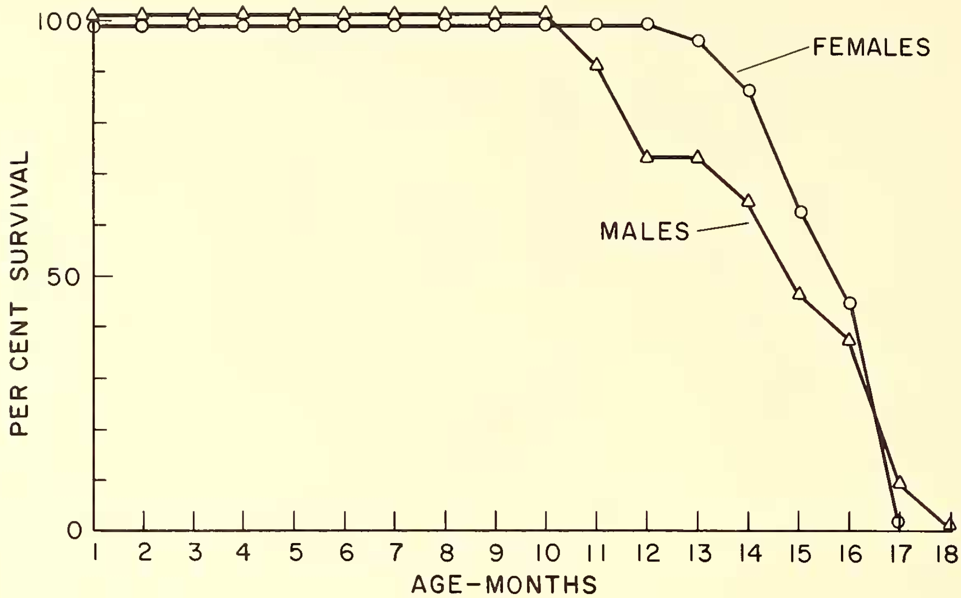
Survival and Autopsy Data on *Cynolebias bellottii*

			MALES
Time interval in months	*Number dying in interval*		*Major gross and microscopic pathology*
1-6	none		
6-7	1	1	Culled
7-10	none		
10-11	1	1	Emaciation. Heterotopic thyroid tissue. Mild reticuloendothelial hyperplasia. Hemosiderosis of spleen.
11-12	2	1	Slight emaciation. Heterotopic thyroid tissue. Chronic renal disease with cyst formation.
		2	Emaciation. Lesions around anus. Heterotopic thyroid tissue. Pharyngitis.
12-13	none		
13-14	1	1	Emaciation. Equilibrium disturbance. Melanism. Neoplasm of gastrointestinal tract. Mild hyperplasia of pharyngeal thyroid tissue.
14-15	2	1	Emaciation. Extensive autolysis. Kidneys with severe vascular sclerosis.
		2	Slight emaciation. Equilibrium disturbance. Lateral curvature of spine. Heterotopic thyroid tissue. Hemosiderosis of spleen.
15-16	1	1	Heterotopic thyroid tissue. Cystic degeneration of spleen.
16-17	3	1	Slight emaciation. Heterotopic thyroid tissue. Intracellular parasitization of pancreas.
		2	Equilibrium disturbance. Heterotopic thyroid tissue. Fatty degeneration of liver. Pharyngitis.
		3	Equilibrium disturbance. Hyperplasia of pharyngeal thyroid tissue.
17-18	1	1	Equilibrium disturbance. Heterotopic thyroid tissue. Hypertrophy of islet tissue of pancreas.
	Total 12		

Time interval in months	Number dying in interval		FEMALES Major gross and microscopic pathology
1-3	none		
3-4	1	1	Accidental death.
4-6	none		
6-7	1	1	Culled
7-12	none		
12-13	1	1	Equilibrium disturbance. Hemorrhage around anus. Mild inflammation of gill filaments.
13-14	2	1	Too autolyzed for histological analysis.
		2	Extreme emaciation. Marked lordosis. Inflammed gastrointestinal tract.
14-15	8	1	Emaciation. Lordosis. Too autolyzed for histological analysis.
		2	Emaciation. Marked lordosis. Degeneration of gill filaments. Inflamed gastrointestinal tract.
		3	Inflammattion and hyperplasia of gill filaments. Inflammation of pharynx, kidneys and ovary.
		4	Too autolyzed for histological analysis.
		5	Too autolyzed for histological analysis.
		6	Slight exophthalmia. Ovarian abscess.
		7	Degeneration of gill filaments. Inclusion cysts in gut wall.
		8	Emaciation. Slight inflammation of gill filaments. Atrophy of body musculature. Large hematopoietic tumor in abdominal cavity, with liver metases. Calcification of renal tubules.
15-16	3	1	Emaciation. Severe lordosis. Exophthalmia.
		2	Slight emaciation. Inflammation and hyperplasia of gill filaments. Possible lymphocystis. Cystic degeneration of spleen.
		3	Extreme emaciation. Lordosis. Exophthalmia. Degeneration of gill filaments. Fatty degeneration of liver. Granulomatous kidney and spleen. Concretions in lower gastrointestinal tract.
16-17	7	1	Too autolyzed for histological analysis.
	Total 23	2	Emaciation. Inflammation and hyperplasia of gill filaments. Concretions and inspissated gastrointestinal cysts.
		3	Slight emaciation. Exophthalmia. Inflammation and hyperplasia of gill filaments. Islet cell tumor. Possible reticuloendothelial hyperpasia.
		4	Slight lordosis. Inflammation and hyperplasia of gill filaments. Fatty degeneration of liver.
		5	Emaciation. Lordosis. Inflammation and hyperplasia of gill filaments.
		6	Slight lordosis. Too autolyzed for histological analysis.
		7	Equilibrium disturbance. Slight lordosis. Heterotopic thyroid tissue. Degeneration of gill filaments. Fatty degeneration of spleen.

by means of the equation of Leslie *et al.* (1955) which compensates for culls and accidental deaths (Text-fig. 1). The form of this curve closely approximates the ideal survival curve of an experimental population exhibiting senescence and a fixed life-span (Comfort, 1961, 1964). For the males of our population, deaths occurred only after the tenth month, after which there was a rapid increase until the 50% mortality level was reached at approximately 14.5 months, with extinction at 18 months. A similar phenomenon occurred with females. Natural deaths began between 12 and 13 months of age, reached 50% at approximately 15.5 months with extinction at 17 months. No statistically significant sexual differences were found at either the midpoint or end of survival, although the first natural female mortality occurred two months later than that of the first male.

2. *Growth.* The monthly growth increments in length and weight are shown in Text-fig. 2. Growth is most rapid from hatching to two months, after which increases in length continue at a slower rate until about 10 months, while weight gain continues to six, seven, or ten months, depending upon the sex. Beyond these time periods, individual fish will often continue to grow, but the values of population samples will either fluctuate or decrease. Such fluctua-



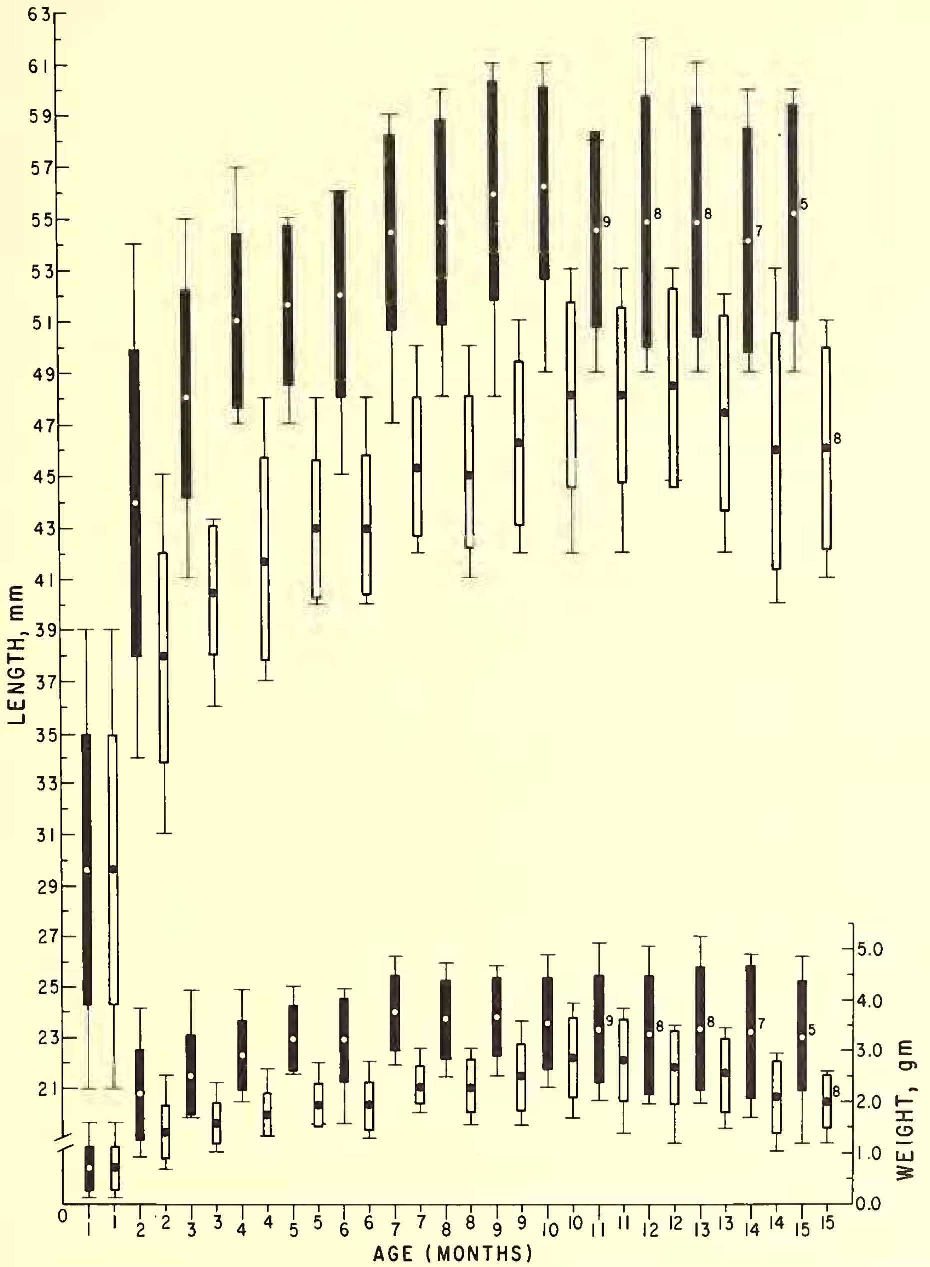
Text-fig. 1. Survival curve for 10 male and 25 female *Cynolebias bellottii* maintained at 22 ± 1° C. throughout life.

tions are usually due to small errors in measuring or to death of animals at either end of the measurement range. The aforementioned factors can contribute to a decreased average length, but such decreases largely reflect the development in aging animals of lordosis, kyphosis, and/or lateral curvature of the spine, thereby reducing the length measurement which is taken as the straight-line distance from the tip of the snout to the end of the caudal peduncle. Decreases in the mean weight with advancing age result from emaciation of individuals in the population samples. When present, emaciation usually occurred one to three months prior to death. Nonetheless, some individuals remained emaciated much longer than one to three months before dying (see Text-fig. 3; Plate I, figs. 1a-2b). It was possible because of their external appearance or behavior (see next section) to follow the growth of certain aging individuals. Only one of three males measured during the last months of its life-span showed a significant decrease in weight; that same individual also decreased in length due to lordosis (Table II).

3. *Egg production.* Eggs were collected from the third to the sixteenth month, but the very small number recovered each month suggested a laboratory artifact rather than decline in capability. One hundred eggs (4.3 eggs per female) were recovered during the third month, but thereafter only 0 to 1 egg was obtained per female, with a range of 3 to 25 total eggs per monthly sample. This small number contrasts markedly with the high egg production in certain stock tanks (790 eggs in nine days by a rapidly dying stock of 18 females, pers. obs., 1963), or by fish maintained in outdoor enclosures (1500 to 2000 eggs by three females in a two months period, Bay, 1966).

4. *Aging and pathology.* When each sample (10 of each sex, unless otherwise stated) was measured, symptoms of overt pathology were also looked for (Text-fig. 3). In brief, these were: equilibrium disturbance, so that the fish swims head down at an increasing angle from the horizontal or lies on the substrate unable to swim without sinking; extended branchiostegals, caused by a swelling of the pharyngeal region internal to these structures; emaciation, evidenced by wasting first above the midlateral line and later along the abdomen (Plate I, fig. 2b); lordosis and kyphosis, a single or double curvature of the spine in the vertical plane; lateral curvature of the spine; exophthalmia; lesions on body; melanism.

Although there was no way to determine whether these external signs were a direct result of physiologic aging, it can be said that these several types of overt pathology increased greatly in frequency as the population grew



TEXT-FIG. 2. Lengths and weights of males and females. At one month, population was not fully sexually differentiated, therefore the same observation on growth data is used for both sexes. The mean, two standard errors of the mean ($2\sigma_M$) and range are represented respectively for males by the open circle, black bar and vertical line, while the same statistics for females are shown by the closed circle, open bar and vertical line. Each observation consists of ten animals, except where noted by a number beside the mean.

older, and, with the possible exception of extended branchiostegals and exophthalmia, may be regarded as symptoms of aging.

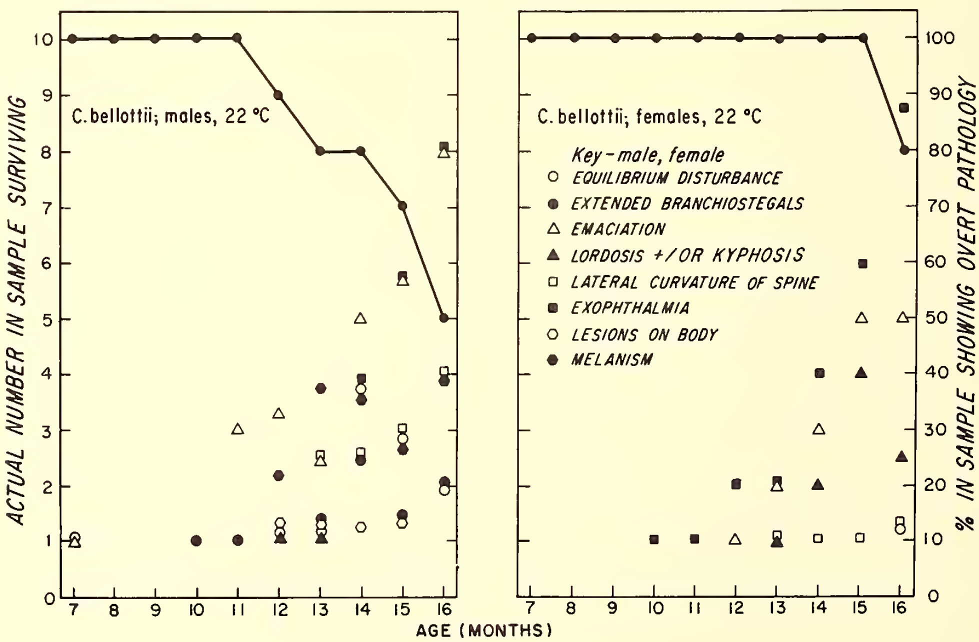
It seems apparent from these data that senescence does not occur simultaneously or synchronously in this species (nor in *C. adloffi, C. wolterstorffi*, pers. obs.), for animals of the same chronologic age may range in appearance from nonsenility to advanced senility (Plate I, figs. 1a-2b). Considerable variation in ratios of soluble/insoluble collagen (the best biochemical "parameter" of aging) between animals of the same age has also been noted (Walford, Sjaarda and Anderson, 1965; Walford, Liu, Troup, and Hsiu, in press).

5. *Pathology.* All the gross symptoms occurred with increasing frequency with age, whereas microscopically only thyroid and gill epithelium showed this age-related distribution (Table III). Because of the characteristic increase in the force of mortality with age, and the rarity of microscopic evidence of an infection, the symptoms of emaciation, equilibrium disturbance, and spinal deformities in the vertical plane were considered to reflect age-related degenerative conditions and not epidemic disease. Changes in the gill filament epithelium were observed in 12 of the 21 females during the last five months of their lives. These changes consisted of inflamed and hyperplastic epithelium (seen also in *C. adloffi* but designated "inflammatory exudate," Walford and Liu, 1965) and a degeneration of the epithelium (Plate II, figs. 3, 4, 5). Gill pathology occurred only in females. It seemed unrelated to any other pathologic findings.

By contrast, hyperplastic and/or heterotopic thyroid tissue was primarily confined to males (10/12 or 83% vs. 1/23 or 4% in females; Tables IV and V). As in the case with the platyfish, *Xiphophorus maculatus,* hyperplasia of the pharyngeal thyroid tissue was often accompanied by heterotopic thyroid tissue in other organs (Baker, 1958a). In the present study, the kidney and spleen of male fish were often infiltrated with thyroid follicles (see Text-fig. 4 for location of major organs; Plate II, fig. 6; Plate III, figs. 7-12; Table IV). No statement can be made as to the relationship of these thyroid conditions to age, since animals were only sampled at death or when moribund. Baker (1958a) reported a definite increase in the number of renal follicles with age. The limited sample of wild specimens of *C. bellottii* available to us showed neither hyperplastic nor heterotopic thyroid tissue (Table V). In *C. adloffi* sectioned during the course of prior studies, no heterotopic tissue was found but in 21 to 40% of the animals the thyroid was hyperplastic (Table V). The other microscopic findings given in Table III have been discussed by Walford and Liu (1965).

Discussion

Under controlled laboratory conditions, our population of *Cynolebias bellottii* reached 50% mortality at 14.5 to 15.5 months; the last of 35



Text-fig. 3. The distribution of overt symptoms of pathology in relation to age. In both portions of the figure, the right ordinate is % in sample showing overt pathology, as indicated by the 8 symbols; the left ordinate is the actual number of survivors in the sample, as indicated by the survival curves.

TABLE II

GROWTH DATA OF INDIVIDUAL MALES

Animal number	*Month 12* Length, Wt.*	*Month 13* Length, Wt.	*Month 14* Length, Wt.	*Month 15* Length, Wt.
1.	58.4; 4.20	58; 4.35	53†; 3.55	55; 3.25
2.	54; 1.95	54; 2.00	53†; 2.20	54; 2.20
3.	....	49; 2.55	49†; 2.50	49; 2.35

† difficulty in obtaining accurate length measurement, usually due to spinal curvature
* length in mm, weight in gms

fish died at 18 months of age. These time periods are longer than previously reported: Boschi (1957) cites approximately nine months as the life-span in nature, but more than a year in the laboratory; Myers (1952) less than one year in the field; Vaz-Ferreira, Sierra de Soriano and Scaglia de Paulete (1964) give 9 to 12 months as the life-span in nature. Boschi (1957) believes that a scarcity of food due to evaporation and reduction in volume of water in their natural habitats is the primary cause of death. Vaz-Ferreira *et al.* (1964) suggests reduction in water volume alone is the prime factor in mortality of the fish. Sternshein (1965) believes a diminishing food supply and high temperatures cause death. Thus, lack of food and physicochemical changes in the environment, caused by the high temperatures of summer in the Southern Hemisphere have been postulated as the causes of death prior to the actual total dessication of the habitat. Therefore, it has been difficult to determine whether under field conditions the fishes of the genus *Cynolebias* are obligate or facultative annuals. It is true that some species of *Cynolebias* are found occasionally in permanent bodies of water (Boschi, 1957; Hildemann and Walford, 1965) and it is known that eggs of *C. bellottii* can develop while kept continuously in water (Scheel, 1962), nevertheless no observations are extant regarding life-span in the wild state in permanent water.

Our own observations with *C. adloffi*, *C. bellottii*, and *C. wolterstorffi* (Walford and Liu, 1965; Liu and Walford, 1966; unpublished date) and that of Boschi (1957) show conclusively that these species can live much longer than a year in the laboratory. They can only be considered facultative annuals. A female *C. adloffi* kept at 16°., the last survivor of the experiment described in Liu and Walford (1966), died when 44 months old; populations of *C. wolterstorffi* maintained at the same temperature had only reached approximately 60% mortality at 30 months, and were still producing viable ova. It may well be that 16°C. is an optimal temperature (58 to 68°F. is indeed given as the best temperature range for *C. bellottii* by Boschi, 1957) not experienced for any long duration in the natural habitat of these fish. Life-span can also be prolonged by restricted feeding. In a preliminary experiment with *C. bellottii* kept at 23 ± 2°C., we fed them three times a week instead of the usual 13 to 14 times; the last male died at 24 months and the last female at 33 months of age. These results are similar to those obtained in rodents subjected to dietary restriction (McCay, 1952). Under more controlled experimentation, it may be possible to show that a combination of high temperatures and decreased food would decrease rather than increase mortality, as suggested by Boschi (1957) and Vaz-Ferreira *et al.* (1964).

In addition to these environmental influences on life-span, certain aspects of the annual fish's ontogeny may play important roles in the determination of its total life-span. Although an egg can develop fully in two to three weeks, some eggs remain without sign of embryonic development for one to two years (maximum of 5½ years, Scheel, 1962). Tertiary developmental arrest can last over six months (Wourms, 1965). Theoretically, therefore, an embryo can hatch after two to three weeks or after six years. Such variation may be inconsequential in nature (except as an adaptation to survival in case of premature rains or extended drought) where each adult population has a life-span corresponding roughly to the availability of water, but in the laboratory, where eggs may be held for varying periods of time prior to hatching, differences in length of time spent as an embryo might subsequently affect life-span after hatching.

Growth is one of the most easily monitored parameters that can be correlated with age. Nevertheless, few laboratory studies giving such data for the latter half of the life-span of fish have been conducted. It is not even fully known whether certain gross signs of aging, such as the fall in weight and length noted with the onset of senility in rodents and man, really occur to a comparable extent in fish.

TABLE III

FREQUENCY OF OCCURRENCE OF GROSS AND MICROSCOPIC PATHOLOGY

	Symptom and/or disease	*Males* (N = 12)	*Females* (N = 23)	*Total* (N = 35)
	cull or accidental death	1	2	3
Gross:	emaciation	7	10	17
	equilibrium disturbance	5	2	7
	lordosis, kyphosis and/or lateral curvature of spine	1	9	10
	exophthalmia	....	4	4
	lesions	1	....	1
	melanism	1	....	1
Microscopic:	hyperplastic and heterotopic thyroid tissue	10	1	11
	hemosiderosis and/or other spleen pathology	3	2	5
	renal diseases	2	1	3
	pancreatic diseases	2	1	3
	liver diseases	1	2	3
	gill filament changes	....	12	12
	inflammation (all organs, except gills)	2	....	2
	reticuloendothelial hyperplasia	1	1	2
	neoplasm	1	1	2
	granulomas	....	1	1
	hemorrhage	....	1	1
	concretions, calcification and cysts	....	3	3
	miscellaneous	....	3	3
	too autolyzed for histological analysis	....	6	6

TABLE IV

DISTRIBUTION OF HETEROTOPIC THYROID TISSUE IN *C. bellottii*

Sex	*No.*	*Age at Death (Mon)*	*Kidney*	*Spleen*	*Mesentery*	*Rete Mirable*	*Gut*
male	1	10-11	..	..	1	..	1
male	2	11-12	2	2	1	..	..
male	2	14-15	1	2	..	1	..
male	1	15-16	1	1	..	1	..
male	2	16-17	..	2	1	..	..
male	1	17-18	1	1	..	..	..
female	1	16-17	1	1	..	..	..

TABLE V

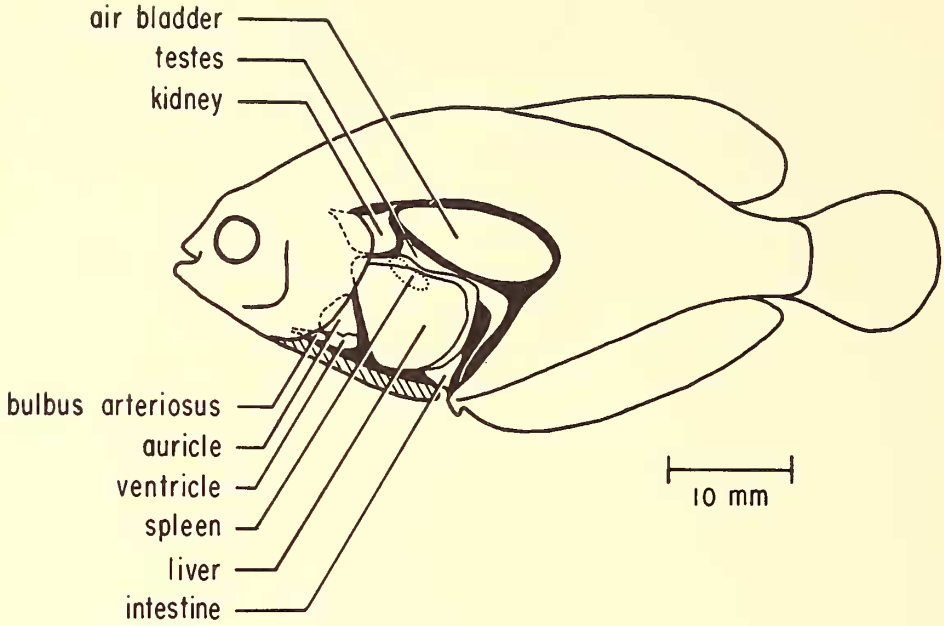
INCIDENCE OF HETEROTOPIC THYROID TISSUE AND THYROID HYPERPLASIA IN VARIOUS SPECIES AND POPULATIONS OF *Cynolebias* KEPT AT DIFFERENT TEMPERATURE REGIMES

Species	*Population. Sex*	*Temp. Regime*	*No. Heterotopic/ Total Analyzed*	*%*	*No. Hyperplastic/ Total Analyzed*	*%*
C.a.	exp., male	22 ± 1°C	0/21	0	7/21	33
C.a.	exp., male	16 ± 1°C	0/14	0	3/14	21
C.a.	exp., female	22 ± 1°C	0/19	0	5/19	26
C.a.	exp., female	16 ± 1°C	0/15	0	6/15	40
C.b.	exp., male	22 ± 1°C	9/12	75	10/12	83
C.b.	exp., female	22 ± 1°C	1/23	4	1/23	4
C.b.	wild, male	unknown	0/4	0	0/4	0
C.b.	wild, female	unknown	0/3	0	0/3	0

Within days after hatching, differences in growth rates of individuals of the same sex were observed in three species of *Cynolebias* we have studied; this rate-inequality continues throughout the life of the animals. Besides genetic differences, length of time spent in developmental arrest as well as the weakened state of some hatchlings may contribute to the differential growth (defined as growth depensation by Brown, 1957; Magnuson, 1962). The wide variability in body size shown by our specimen, and by Bay's specimens of *C. bellottii* (pers. comm.) is indicative of the difficulty in exactly duplicating experimental results. Sex and behavior also play roles in determining size differences; the inhibiting effect on growth by hierarchies and other dominance relations has been clearly shown by Brown (1957), Kinne (1960), Magnuson (1962), and Yamagishi (1962). In addition to reduction in growth and increased susceptibility to stress, the smaller and most often subordinate members of a population may also show enhanced susceptibility to diseases and parasites (Erickson, 1967; Barrow, 1955).

The effects of sex on growth are not as drastic in the oviparous annuals as in viviparous cyprinodonts or poecillids. Among the latter, males usually exhibit a sharp decrease in growth rate after sexual maturity (Felin, 1951; Wellensiek, 1953). The relationship between sex, sexual maturity, and growth in livebearers has been clarified by the extensive studies of Peters (1964). Compared to poecilliids, annuals show fewer changes in specific growth rate since sexual maturity occurs very early (18 days or fewer for pond-reared *C. bellottii,* Bay, pers. comm.), and annuals are probably not reproductively senescent at death. But with females of both annuals and poecillids, a large percentage of the weight is determined by the number and state of development of the ova or embryos, factors which are in turn dependent upon spawning or mating frequency. Aronson (1951) calculated that the differences in weight gain between intact and castrated female cichlids were attributable to the loss of eggs.

In order to apply such compensation to the weight data for females, 20 developing eggs of *C. bellottii* were measured and weighed; average egg diameter was 1.35 mm, average weight 1.3 mg. Using the data for egg production from a stock population mentioned previously (see section on egg production), we calculated that each female laid 18 eggs or 23.4 mg per day. In 30 days, this would total 0.7 gm or approximately 30% of the total body weight of a 2.0 gm female. Therefore, weight loss through egg oviposition can more than compensate for sexual



TEXT-FIG. 4. Location of some major organs in the male *of C. bellottii.*

differences in weight. Even if this rough estimate were incorrect by 50%, average female weight would match that of males up to the fifth month, and thereafter would surpass it.

Since annuals usually require live food, a nutritious and reliable supply of such food must be on hand. Adult *Artemia* are nutritious, are generally available, and in addition are relatively pathogen-free; however, their limited survival time in fresh water makes it impossible to maintain brine shrimp in each aquarium throughout the diel period in quantity comparable to that of field or pond situations. Feeding is additionally complicated by differences in food consumption at various ontogenetic stages, under different environmental conditions, and by the amount of manipulation of the individual during the different techniques used in measurement of growth parameters (Kinne, 1960). Even if these difficulties could be overcome, certain time-related effects would remain. As the number of fish in the aging population declines, more space and possibly more food becomes available to the survivors, unless the amount of tank-space were continually decreased. It is largely impractical to do so. With decreased population density, a decrease in concentration of metabolites and therefore in metabolic inhibition of growth (Kawamoto, 1961; Richards, 1958, in tadpoles) might obtain. Thus, the tendency to decline in growth rate due to age could be partially offset by the stimulating effects of increasing the amount of space available to each surviving fish.

If individuals of different size-classes show differences in mortality rates, the skewed population average could also mask any differences in growth (Silliman and Gutsell, 1958). In addition, we have observed that older animals eat less than younger animals. Whether obligatory or facultative in nature, this decreased food intake might have dual effects: either negating or decreasing the growth-promoting influence of more space due to fewer survivors, or leading to a prolongation of life-span. Under appropriate conditions, dietary restriction has been shown to prolong life in various invertebrates, rodents, and poeciliids (Comfort 1960, 1963, 1964). According to our preliminary observations, the same effect obtains with *C. bellottii.*

Our growth curves for *C. bellottii* are qualitatively similar to those observed for other small, relatively short-lived fish (*C. adloffi*, Walford and Liu, 1965; Liu and Walford, 1966; *Cyprinodon macularius*, Kinne, 1960; various poeciliids, Comfort, 1960, 1963; Felin, 1951; Peters, 1964; and Wellensieck, 1953). Some preliminary data on *C. bellottii* in semi-natural conditions have been obtained by Bay (pers. comm.). Eighteen-day-old, sexually mature *C. bellottii*, exposed since hatching to temperatures ranging from 73 to 90°F. (22.5-32.5°C.) in a 15 x 20 foot soil-bed pond, had an average total length of 47.1 mm (sample of 10, range 42-50 mm) for males, 35.3 mm total length (sample of 9, range 37-41 mm) for females, and average weights of 1.51 and 0.68 grams respectively. At 26 days of age, 10 males and 10 females from the same population displayed average total lengths of 50.6 mm (38-59 mm) for males, 40.5 mm (26-47 mm) for females and average weights of 2.10 and 1.01 grams. Comparable lengths (expressed in standard lengths) and weights are not achieved by laboratory-reared animals until at least two to three months of age (Text-fig. 2). Prior laboratory studies with sexually mature *C. adloffi* (a closely related species) showed that faster growth occurred at a lower temperature (16°C. vs. 22°C.), yet these pond-reared *C. bellottii* were able to attain a very large size in less than one month at relatively high temperatures (22 to 32°C.). Our current populations of *C. bellottii* have been maintained since hatching at 15°C. and 20°C. The 20°C. fish show initially a slightly more accelerated growth than those at 15°C., but by four to eight months of age the latter have surpassed those at the higher temperature. Perhaps subjecting annuals to a high temperature prior to sexual maturity may promote growth, while exposure to the same high temperature after sexual maturity does not.

No real comparison can be made of the vastly different biological and physical parameters extant in the aquarium as against pond situations with regard to growth. As an example, the longer photo-period of summer (compared to eight hours in the laboratory), would permit the fish more time to feed on the constantly available food in the pond. Since increased food consumption and increased photo-period both promote growth (Gross, Roelofs, and Fromm, 1965), these factors alone may be sufficient to cause the growth discrepancy between laboratory and field populations.

Any attempt to equate size with age would be dangerous, since there are no independent correlates of age in these fish. Annuli or other time-related indications of growth are absent in fish that usually live less than a year in nature. Biochemical correlates of aging could probably at best only give a very approximate index of age, as indicated by our work with soluble—insoluble collagen ratios in *C. bellottii* (Walford, Liu, Troup, and Hsiu, in press).

The following factors may be involved in the small numbers of eggs we recovered. Boschi (1957) has stated that an abundant supply of food is necessary for continued spawning; twice daily feedings of the stated amounts may have been insufficient to meet the requirements of prolonged spawning. Bay (1966) obtained less than 0.1% hatch for overwintering eggs of *C. bellottii*. A high degree of egg mortality could also account for our results.

In this study increased mortality, decreased growth rates, and visible signs of pathology appeared to be the only correlates of aging (Text-figs. 1-3). These phenomena are, however, all expressed primarily on the population level, so that any one individual may or may not exhibit them. Only one of three males showed a significant loss of weight during the last months of his life-span (Table II). The 44-month-old female survivor previously mentioned had maintained her weight, while the longest-lived specimen at 16°C. showed a consistent and significant weight gain up to death and did not manifest overt symptoms of senility (Walford and Liu 1965). Again, a comparison of four specimens of the same age shows that senescence does not occur synchronously (Plate I, figs. 1a-2b).

In previous observations with *C. adloffi* (Walford and Liu, 1965), equilibrium disturbance had been releated to a displacement of the swim-bladder or to a reduction in the volume of gas in the bladder by hyperplastic reticuloendothelial tissue. It is not known whether this hyperplasia is correlated to age or to stress. The stress of continued darkness may provoke reactions in reticuloendothelial and adreno-cortical tissues (Rasquin and Rosenbloom, 1954). Extended branchiostegals are usually an indication of thyroid disorders. Matty, Wenzel, and Bardach (1958) have produced exophthalmia by injections of thyroxine and triiodothyronine. Van Duijn (1967) has reviewed other causes of this disease. Emaciation, often accompanied by lordosis and/or kyphosis and equilibrium disturbance (Plate I, fig. 2b), has probably the same relationship to senility in fish as it has in mammals. Lordosis (or kyphosis) in *Astyanax* appeared to be due to fibrosis of the vertical arches, caused by a malfunction of calcium metabolism (Rasquin and Rosenbloom, 1954). Comfort (1960) attributes lordosis to fast growth at high temperatures and not simply to old age. Harrington (1967) reported kyphosis in the self-fertilizing hermaphrodite fish, *Rivulus mar-*

moratus, when exposed to dim light, fresh water, and high temperatures (30°C.) or to dim light, sea water, and low temperatures (18 to 20°C.).

Hyperplasia of the pharyngeal thyroid tissue and a high incidence of heterotopic thyroid tissue were the most striking feature of microscopic pathology in male *C. bellottii*. Baker (1958a, 1958b, 1959) and Baker-Cohen (1961) suggested that heterotopic thyroid tissue was common in freshwater teleosts, that this tissue showed increased development under iodine-poor conditions (0.5-1.0 μg/l.) and that such development could be prevented by the addition of potassium iodide to the aquarium water.

However, it is difficult to attribute low iodine content in the water as the cause for thyroid pathology in our fish. The tap water for our laboratories comes from a source relatively extremely rich in iodine (23 μg/l., according to the 1964-65 averages of the Los Angeles Department of Water and Power). In addition, after the appearance of extended branchiostegals and histological evidence of hyperplastic and heterotopic thyroid tissue, we periodically added potassium iodide to the aquarium water and to the medium in which the food (adult *Artemia*) were maintained. If iodide-binding substances were present, resulting in a lowering of the iodine content of the water, both sexes should have been equally affected, whereas the condition was noted almost exclusively in males (Tables III, IV, and V). The few, field-collected male and female animals available to us showed neither hyperplastic nor heterotopic thyroid tissue, while the related species *C. adloffi* showed under similar laboratory conditions a moderate and non-sex-related degree of hyperplasia of the pharyngeal thyroid (Table V). No heterotopic thyroid tissue was found in *C. adloffi*. Baker (1958a) noted a higher frequency of heterotopic thyroid tissue in the kidneys of male *Xiphophorus maculatus* of the FU strain and in the kidneys of females of the BH strain.

Growth of males was not markedly decelerated in comparison to that of females, so we assume thyroid activity was normal or possibly hyperactive during most of their life-span. No developmental or gonadal retardation was observed such as that reported by Baker (1958a) or La Roche *et al.* (1966).

Changes of the gill filament epithelium was another microscopic feature which increased with advancing age. We can offer no reason why this occurred only with females. Degeneration of the gill epithelium (4/12 fish; Plate II, fig. 4) has not been reported previously in the literature, whereas inflammation and hyperplasia of the epithelium (8/12 cases; Plate II, fig. 5) so as to cause adhesion or fusion of the gill filaments have been noted by Rasquin and Rosenbloom (1954) and Burrows (1964). The first authors associated this epithelial hyperplasia with adrenal insufficiency in dark-stressed fish. Burrows, by subjecting chinook salmon fingerlings to increasing concentrations of ammonium hydroxide for periods of six weeks obtained increased hyperplasia of the filaments. Despite filtration and partial monthly replacement of the water in our tanks, a gradual increase in ammonia from excretory products could have occurred and produced the hyperplasia we observed. A direct effect of suspended peat particles on the gills can probably be ruled out, since even at very high turbidities (100,000 ppm and greater), clogging of the gill filaments rather than histological changes are observed (Wallen, 1951).

Summary and Conclusions

1. Various aspects of the biology of an annual fish, *Cynolebias bellottii* Steindachner, were investigated to provide baseline information necessary for studies on the physiology of aging. This species has proven to be a suitable experimental animal for gerontological studies.

2. Maintained since hatching at 22 ± 1°C., on an eight-hour photo-period with twice daily feedings of live, adult *Artemia* (after the first four weeks), the males reached 50% mortality at 14.5 months, with extinction at 18 months. Females attained 50% mortality at approximately 15.5 months, extinction at 17 months. Survival curves for both sexes strongly suggested the occurrence of senescence in these fish. Factors affecting the life-span of annuals are discussed, including the phases of their life cycles which may affect their rates of aging.

3. Growth was most rapid from hatching to two months, after which increases in length continued at a slower rate until about 10 months, while weight gain continued to six-seven or ten months, depending upon the sex. Beyond these periods, individual fish may either continue to grow, show no increase in growth, or lose weight and/or decrease in length. The population averages for length and weight tended to decline with advanced age. Emaciation increased with age in both sexes. Growth was significantly greater in males.

4. No conclusions about reproductive senescence could be made on the basis of the observed data on egg production. The low number of eggs recovered was attributed to a laboratory artifact.

5. Overt symptoms of pathology were monitored monthly and shown to increase with age.

Whenever possible, each animal was autopsied; emaciation, equilibrium disturbance, and spinal curvature were the major gross symptoms. On the microscopic level, hyperplastic and heterotopic thyroid tissue and gill filament changes were most frequent. Manifestations of atypical thyroidism were almost exclusively confined to males (75 to 83% vs. 4% in females) while inflamed, hyperplastic and degenerated gill filaments were only seen in females.

ACKNOWLEDGMENTS

We would like to thank Dr. Vladimir Walters, Richard Haas, and Bruce J. Turner for critical reading of the manuscript. Much appreciation is due Professor Rogelio Lopez for sending the parental stock of *C. bellottii* from Argentina, and the Sanitary Engineering Division, Department of Water and Power, City of Los Angeles, for supplying the 1964-65 water analyses.

LITERATURE CITED

ARONSON, L. R.

1951. Factors influencing the spawning frequency in the female cichlid fish *Tilapia macrocephala* (Bleeker). Am. Museum Novitates No. 1484:1-26.

BAKER, K. F.

1958a. Heterotopic thyroid tissues in fishes. I. The origin and development of heterotopic thyroid tissue in platyfish. J. Morph., 103: 91-134.

1958b. Heterotopic thyroid tissues in fishes. II. The effect of iodine and thiourea upon the development of heterotopic thyroid tissue in platyfish. J. Exp. Zool., 138:329-354.

1959. Heterotopic thyroid tissues in fishes. III. Extrapharyngeal thyroid tissue in Montezuma swordtails, a guppy and a cherry barb. Zoologica, 44 (4):133-140, 1 pl.

BAKER-COHEN

1961. The role of the thyroid in the development of platyfish. Zoologica, 46(4):181-222, 9 pls.

BARROW, J. H.

1955. Social behavior in fresh-water fish and its effect on resistance to trypanosomes. Proc. Nat. Acad. Sci., 41(9):676-679.

BAY, E. C.

1966. Adaptation studies with the Argentine pearl fish, *Cynolebias bellottii*, for its introduction into California. Copeia, (4): 839-846.

BOSCHI, E.

1957. Argentine pearl fish. English translation from Spanish by W. Vorderwinkler. T. F. H. Publ. Inc., Jersey City, N. J., 23 pp.

BROWN, M. E.

1957. Experimental studies on growth. *In* Physiology of fishes, M. E. Brown (ed.). N. Y., Acad. Press., 1:361-400.

BURROWS, R. E.

1964. Effects of accumulated excretory products on hatchery-reared salmonids. U. S. Bureau of Sport Fisheries and Wildlife, Research Rept., 66:1-12.

COMFORT, A.

1960. The effect of age on growth-resumption in fish (*Lebistes*) checked by food restriction. Gerontologia, 4:177-186.

1963. Effect of delayed and resumed growth on the longevity of a fish (*Lebistes reticulatus* Peters) in captivity. Gerontologia, 8:150-155.

1964. Aging: the biology of senescence. London, Routledge and Kegan Paul, 365 pp.

ERICKSON, J. G.

1967. Social hierarchy, territoriality and stress reactions in sunfish. Physiol. Zool., 40 (1):40-48.

FELIN, F. E.

1951. Growth characteristics of the poeciliid fish *Platypoecilus maculatus*. Copeia, (1): 15-28.

GROSS, W. L., E. W. ROELOFS, AND P. O. FROMM

1965. Influence of photoperiod on growth of green sunfish, *Lepomis cyanellus*. J. Fish. Res. Bd. Canada, 22(6):1379-1386.

HARRINGTON, R. W., JR.

1967. Environmentally controlled induction of primary male gonochorists from eggs of the self-fertilizing hermaphroditic fish, *Rivulus marmoratus* Poey. Biol. Bull., 132(2):174-199.

HILDEMANN, W. H., AND R. L. WALFORD

1963. Annual fishes – promising species as biological control agents. J. Trop. Med. & Hygiene, 66:163-166.

KAWAMOTO, N. Y.

1961. The influence of excretory substances of fishes on their own growth. Prog. Fish-Culturist, 23(2):70-75.

KINNE, O.

1960. Growth, food intake, and food conversion in a euryplastic fish exposed to different temperatures and salinities. Physiol. Zool., 33(4):288-317.

LA ROCHE, G., A. N. WOODALL, C. L. JOHNSON, AND J. E. HALVER

1966. Thyroid function in the rainbow trout (*Salmo gairdnerii Rich.*) II. Effects of thyroidectomy on the development of young fish. Gen. & Comp. Endoc., 6(2): 249-266.

Leslie, P. H., J. S. Tener, M. Vizoso, and H. Chitty
1955. The longevity and fertility of the Orkney Vole, *Microtus orcadensis,* as observed in the laboratory. Proc. Zool. Soc. Lond., 125:115-125.

Liu, R. K., and R. L. Walford
1966. Increased growth and life-span with lowered ambient temperature in the annual fish, *Cynolebias adloffi.* Nature, 212 (5067):1277-1278.

McCay, C. M.
1952. *In* Lansing, A. Z. (ed.). Problems of aging. Baltimore, Williams and Wilkins Company, 1061 pp.

Magnuson, J. J.
1962. An analysis of aggressive behavior, growth, and competition for food and space in medaka (*Oryzias latipes*) (Pisces, Cyprinodontidae) Can. J. Zool., 40:313-363.

Matty, A. J., D. Menzel, and J. E. Bardach
1958. The production of exophthalmos by androgens in two species of teleost fish. J. Endocrinol., 17:314-318.

Myers, G. S.
1952. Annual fishes. Aquarium J., 23(7):125-141.

Peters, G.
1964. Vergleichende Untersuchungen an drei Subspecies von *Xiphophorus helleri* Heckel (Pisces). Zt. Zool. syst. Evolutionsforschung., 2:185-271.

Rasquin, P.
1949. The influence of light and darkness on thyroid and pituitary activity of the characin *Astyanax mexicanus* and its cave derivatives. Bull. Amer. Mus. Nat. Hist., 93:501-531.

Rasquin, P., and L. Rosenbloom
1954. Endocrine imbalance and tissue hyperplasia in teleosts maintained in darkness. Bull. Amer. Mus. Nat. Hist., 104(4):365-425, 23 pls.

Richards, C. M.
1958. The inhibition of growth in crowded *Rana pipiens* tadpoles. Physiol. Zool., 31:138-151.

Rose, S. M.
1959a. Population control in guppies. Amer. Midl. Nat., 62(2):474-481.
1959b. Failure of survival of slowly growing members of a population. Science, 129 (3355):1026.

Scheel, J. J.
1962. Factors governing development of killie eggs. Pt. II. Tropicals, 7(2):9-15.

Silliman, R. P., and J. S. Gutsell
1958. Experimental exploitation of fish populations. U. S. Fish and Wildlife Serv., Fish. Bull., 58(133):215-252.

Sternshein, I.
1965. *Cynolebias* in the wild. J. Amer. Killifish Assoc., 2(2):3-5.

Van Duijn, C., Jr.
1967. Diseases of fishes. London, Illiffe Bks. Ltd., 309 pp.

Vaz-Ferreira, R., B. Sierra de Soriano, and Scaglia de Paulete
1964. Eco-etología de la reproducción en los peces del género *Cynolebias* Steindachner, 1876. Arch. Soc. Biol. Montevideo, 26: 44-49.

Walford, R. L., R. K. Liu, G. M. Troup, and Julia Hsiu
Alterations in soluble/insoluble collagen ratios in the annual fish, *Cynolebias bellottii,* in relation to age and environmental temperature. Exp. Geront., *in press.*

Walford, R. L., J. R. Sjaarda, and R. E. Anderson
1965. Life-span and soluble–insoluble collagen (hydroxyproline) ratios in normal and irradiated hamsters. Exper. Geront., 1:117-125.

Wallen, I. E.
1951. The direct effect of turbidity on fishes. Okla. Agr. & Mech. Coll., Bul. Biol. Ser. (2), 48(2):1-27.

Wellensiek, U.
1953. Die Allometrieverhältnisse und Konstruktionsänderung bei dem kleinsten Fisch im Vergleich mit etwas grösseren verwandten Formen. Zool. Jb. (Anat.), 73:187-288.

Wourms, J. B.
1965. Comparative observations on the early embryology of *Nothobranchius taeniopygus* (Hilg.) and *Aplocheilichythys pumilis* (Blgr.) with especial reference to the problem of naturally occurring diapause in teleost fishes. Annual Rept, 1964, East African Freshwater Fisheries Research Organization. Appendix H:68-73.

Yamagishi, H.
1962. Growth relation in some small experimental populations of rainbow trout fry, *Salmo gairdnerii* Richardson with special reference to social relations among individuals. Jap. J. Ecol., 12(2):43-53.

EXPLANATION OF THE PLATES

Plate I

Anesthesized 14-month-old specimens of *C. bellottii,* photographed out of water. All X 1.5.

Fig. 1a. Male with non-senile appearance.

Fig. 1b. Male showing slight emaciation above the midlateral line.

Fig. 2a. Female with non-senile appearance.

Fig. 2b. Female showing advanced lordosis, emaciation and exophthalmia.

Plate II

Fig. 3. Normal gill epithelium. X 100.

Fig. 4. Degenerated gill epithelium. X 100.

Fig. 5. Inflamed and hyperplastic gill epithelium. X 100.

Fig. 6. Composite photomicrograph of a sagital section of a male. Note large number of thyroid follicles in pharyngeal area (P) and heterotopic thyroid follicles in kidney (K) and spleen (S). Most of the colloid was not retained during sectioning so that the follicles appear as white circular spaces. AD equals anterior dorsal, PV equals posterior ventral position.

Plate III

Fig. 7. Pharyngeal area of a *C. bellottii* female, showing the normal distribution of thyroid follicles. X 240.

Fig. 8. Pharyngeal area of a male with hyperplasia of the thyroid. X 240.

Fig. 9. Normal kidney of a female. X 240.

Fig. 10. Heterotopic thyroid follicles in the kidney of a male. X 240.

Fig. 11. Normal spleen of a female. X 240.

Fig. 12. Heterotopic thyroid follicles in the spleen of a male. X 240.